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13. ABSTRACT (Maximum 200 Words) The HER-2/ <i>neu</i> oncogene is amplified and overexpressed in 25-30% of patients with breast cancer. The gene encodes a protein receptor found on the surface of breast cancer cells in this subset of patients. Four novel monoclonal antibodies, 5A7, 8H11, 11F11, and 10H8, were isolated and characterized in this work. The ultimate aim of this work was to develop target specific gene therapy for breast cancer. To further that aim, we used a rapid radioimmunoassay to detect antibodies which could bind to live human breast cancer cells and trigger internalization into those cells. Monoclonal antibodies 8H11 and 10H8 were found to have this specific property. The next major step was the cloning of the variable region gene sequences of these monoclonal antibodies from the hybridoma cell lines isolated for antibody production. The ability to design degenerate primers for specific amplification of the DNA encoding these variable regions allowed for the cloning, sequencing, and then splicing of the variable heavy (VH) with the variable light (VL) genes. The subsequent single chain variable fragment (scFv) was cloned and determined to approximate the antigen binding site of the parent immunoglobulin. An attempt to characterize the protein product of the scFv gene of 10H8 met with problems during protein expression. Therefore, the final aim of establishing target specificity to retroviral vectors ended with an unsuccessful endpoint which exceeded the ability of the trainee to complete in this funding period.					
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....(Follows Introduction as Materials and Methods)	5
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	13
Appendices.....	14-28

INTRODUCTION

Targeted therapy for human cancers requires the use of well-characterized reagents that specifically recognize tumor cells in the host background (1). Recent identification of differences in the genotype and molecular structure of cancer cells has provided an opportunity to develop targetable reagents for the specific therapy of selected human cancers (2, 3, 4, 5). Amplification of the *HER-2/neu* oncogene in human breast cancers, endometrial cancers, ovarian cancers, gastric cancers, and salivary gland carcinomas provides a potential molecular target for these cancers (6, 7, 8, 9, 10). *HER-2/neu* gene amplification is associated with overexpression of the p185^{HER-2} protein. Amplification and overexpression is correlated with a shorter time to relapse and a shorter overall patient survival (9, 10). A humanized monoclonal antibody, HerceptinTM, has been used to successfully treat some women with *HER-2/neu* overexpressing breast and ovarian cancers, demonstrating that p185^{HER-2} can be used to selectively target human cancers (11, 12). While outcomes have improved for patients treated with HerceptinTM, more effective classes of antibody-based therapies need to be developed to capitalize on cancer specific antigen expression.

Several groups have tested p185^{HER-2} overexpressing cells as a target for gene therapy (13, 14, 15, 16). Targeted delivery of genes has been attempted by altering cognate receptors on virions with cellular receptor ligands and single-chain antibodies (17, 18, 19, 20). Although these vectors have successfully bound p185^{HER-2} overexpressing cells, none have resulted in significant rates of gene transduction.

Because the successful gene therapy vector will require not only cell targeting, but internalization of the vector and expression of a therapeutic gene, it is important to evaluate as many of these steps as possible prior to construction of the vector. To this end, we isolated several anti-p185^{HER-2} monoclonal antibodies and characterized their internalization potential using a rapid, quantifiable radioimmunoassay. Two of the four p185^{HER-2} monoclonal antibodies bound to viable, intact target cells, were internalized and then trafficked through an endolysosomal pathway. These antibodies are expected to be useful in targeting treatment vectors that require internalization for therapeutic effect.

MATERIALS AND METHODS

Production and isolation of monoclonal antibodies to the extracellular domain (ECD) of p185^{HER-2} involved the use of recombinant ECD^{HER-2} protein or viable *HER-2/neu* overexpressing cell lines for immunization of BALB/c mice, and screening of hybridomas with ELISA and immunocytochemical assays. The monoclonal antibodies were purified by affinity chromatography. The specificity of the antibodies was determined with Western immunoblot analyses, immunoprecipitation assays, and immunohistochemistry. The ability of monoclonal antibodies to specifically recognize viable *HER-2/neu* overexpressing cell lines was evaluated with fluorescence microscopy and flow cytometry. Cellular trafficking of monoclonal antibodies was characterized with radioimmunoassay methods.

Protein production and purification

A truncated p185^{HER-2} protein was synthesized and injected into mice as the initial immunogen for generating anti-p185^{HER-2} monoclonal antibodies. The cDNA of the *HER-2/neu* gene was cut between the NcoI and SphI sites, yielding a portion of the open reading frame which includes the initiation codon extending to the transmembrane domain (21). The 2-kilobase DNA insert was ligated into the multiple cloning site immediately downstream of an inducible *trp-lac* promoter of a pTrcHisA expression vector (Invitrogen, San Diego, CA). Successful in-frame subcloning of the *HER-2/neu* gene was confirmed by restriction fragment analysis and direct DNA sequencing (Amersham Pharmacia Biotech, Piscataway, NJ). TOP10 *E. coli* (Invitrogen) were transformed using the *HER-2/neu* expression vector and induced to produce a fusion protein

containing a leader peptide with six contiguous histidine residues followed by the ECD^{HER-2}. Proteins were purified by conditions using metal chelate affinity chromatography on Ni²⁺-NTA-agarose as suggested by the manufacturer (QIAGEN, Valencia, CA).

Cell culture

In addition to recombinant ECD^{HER-2} protein, p185^{HER-2} overexpressing intact cells (NIH/189 and SKBR-3 cells) were used to immunize BALB/c mice. NIH/189 and NIH 3T3 cells were used as sources of intact or solubilized p185^{HER-2} protein for immunologic assays. The NIH/189 cell line, which has been described previously as a p185^{HER-2} overexpressor, was a generous gift from C. Richter King (22).

NIH 3T3, NIH/189, and A431 human epidermoid carcinoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies). The SKBR-3 human breast adenocarcinoma cell line was grown in McCoy's 5A media supplemented with 10% FBS and penicillin/streptomycin. Normal HMEC (human mammary epithelial cell) cells were grown in media formulated by the supplier of the cells (Clonetics, San Diego, CA). These cells were used as non-p185^{HER-2} expressing controls for Western blots and immunoprecipitations. Sp2/0-Ag14 mouse myeloma cells were grown in RPMI-1640 media supplemented with sodium pyruvate, L-glutamine, penicillin/streptomycin, and 15% FBS prior to cell fusion.

Immunization

Two sets of 8-9 week-old female BALB/c mice were immunized with either protein or live cells as immunogens. Three female BALB/c mice received three sequential immunizations of 100 µg, 50 µg, and 50 µg each of the truncated HER-2/*neu* protein. The first immunization of ECD^{HER-2} was an intraperitoneal injection of protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). At weeks 3 and 5, mice were given two immunizations of 50 µg of protein mixed with Freund's incomplete adjuvant (Sigma). The mouse that yielded two monoclonal antibodies (5A7 and 11F11) in this work was given three further boosts of 50 µg ECD^{HER-2}.

Mice were also inoculated with live p185^{HER-2}-overexpressing cells in order to ensure the widest range of monoclonal antibodies against all potential ECD^{HER-2} epitopes, including glycosylated regions. Initial immunizations were intraperitoneal injections of 2-5 x 10⁶ SKBR-3 human breast cancer cells in serum-free RPMI-1640 media over a 7 month period. The mouse that yielded monoclonal antibodies 8H11 and 10H8 had another boost of 5x10⁶ NIH/189 cells in Freund's incomplete adjuvant injected intraperitoneally and 1x10⁶ cells injected subcutaneously. The final immunizations before fusion were an intraperitoneal boost of 10x10⁶ NIH/189 cells in PBS and an intravenous injection of 50 µg of ECD^{HER-2} protein in a buffer containing 0.1M NaH₂PO₄ and 10 mM TrisHCl, pH 8.0.

Hybridoma production and monoclonal antibody screening

Spleen cells from BALB/c mice immunized with either protein or cells were fused to Sp2/0-Ag14 mouse myeloma cells to generate hybridoma cells (23). Cells were fused with polyethylene glycol in a method modified from Köhler and Milstein (24). Hybridoma cells were selected for anti-p185^{HER-2} antibody production by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.

Protein and cell ELISA

ELISA was used as the primary method to screen hybridoma cell clone supernatants for secretion of antibodies. ECD^{HER-2} (250 ng/well) protein was coated onto 96-well plates in PBS. After blocking in 3% bovine serum albumin (BSA), 50 μ L of supernatant were incubated from 1-2 hours at room temperature. After washing 3 times with PBS, a 1:2000 dilution (in 3% BSA) of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) was incubated for 30 minutes at room temperature. Positive wells were visualized by *o*-phenylene diamine (OPD) substrate (Sigma) and read at O.D. 490 nm on an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

To ensure detection of antibodies recognizing conformational and polysaccharide epitopes, cell ELISA using NIH3T3 and NIH/189 cells was employed to detect such clones. Cells (1×10^4 /well) were fixed using 100% methanol onto 96-well Linbro Titertek (ICN, Irvine, CA) plates after overnight attachment onto 1.5% gelatin (Difco, Detroit, MI). The protocol for protein ELISA was used for cell ELISA.

Immunohistochemistry

Frozen and paraffin-embedded surgical biopsies of both known low and high p185^{HER-2} expressor breast cancers were tested for binding of p185^{HER-2} by these four monoclonal antibodies. The use of human tissue was approved prior to this work by the Institutional Review Board of the USC School of Medicine.

Frozen tissue section immunohistochemistry was used as a secondary screening method to detect positive hybridomas that secreted antibodies to intact p185^{HER-2} from human tissue. p185^{HER-2} in tissue sections were detected by the peroxidase-anti-peroxidase technique as previously described (25).

Breast cancer cases embedded in paraffin as a single multi-tumor block were also tested for p185^{HER-2} recognition. Formalin-fixed breast cancer biopsies with known low and high expression of p185^{HER-2} protein were embedded in a specified matrix in a single paraffin block (26). Monoclonal antibodies purified from ascites were used at equal concentrations to compare localization in a single specimen.

Western blots and immunoprecipitation

Monoclonal antibodies were used to probe for full-length p185^{HER-2} in total protein lysates of both mouse and human cell lines loaded equally in SDS-PAGE. The method used has been previously described (27).

Immunoprecipitations demonstrated the ability of monoclonal antibodies to bind to full length HER-2/*neu* in soluble detergent lysates of mouse and human cell lines. The immunoprecipitation method used has been previously described (28). A rabbit polyclonal antibody (R60) previously reported for use in p185^{HER-2} Western blots was used (10). A 1:2000 dilution of R60 polyclonal sera in 10% goat serum was used to detect the C-terminus of immunoprecipitated p185^{HER-2} protein in the blot.

Flow cytometry

A prerequisite for the determination of cellular trafficking of monoclonal antibody-antigen complexes is the binding of monoclonal antibody to its target on the surface of p185^{HER-2} overexpressing cells. Fluorescence-activated cell sorting (FACS) was used to determine ligation of monoclonal antibodies to native p185^{HER-2} on the surface of SKBR-3 cells. The flow cytometry method has been previously described (29). Cells were analyzed on a Coulter Elite ESP Cell Sorter (Beckman Coulter, Miami Lakes, FL). Mouse IgG isotype control was used as a negative control for FACS (Zymed).

Radioimmunoassay

A method to detect internalization and intracellular trafficking of monoclonal antibodies directed against cell-surface antigens was used as previously described to characterize anti-p185^{HER-2} monoclonal antibodies with this potential (30, 31). Monoclonal antibodies 8H11 and 10H8 were labeled with ¹²⁵I (Amersham Pharmacia) using the Iodo-Gen method (Pierce). NIH 3T3 and NIH/189 cells were pulsed with labeled monoclonal antibodies and chased with 1% BSA/RPMI-1640. Supernatant, acid-releasable surface-bound, and intracellular label were collected and counted on a Cobra Automated Gamma Counter (Packard, Meriden, CT). The supernatant fraction was further divided between 25% TCA precipitable and 25% TCA soluble components representing shed, intact monoclonal antibodies and degraded monoclonal antibodies, respectively. These fractions were collected at times of 0, 1, 4, 19, and 24 hours. All timepoint determinations were corrected for non-specific initial binding (T=0 hours) by subtracting counts in fractions after surface blocking by unlabeled monoclonal antibodies.

RESULTS

Four monoclonal antibodies have been isolated and characterized for their individual characteristics in standard immunoassays and in an internalization assay to determine their potential for use with internalization-dependent therapies.

Production and characterization of monoclonal antibodies. A 70 kDa, truncated ECD^{HER-2} protein was isolated from inclusion bodies of bacteria which had been transformed by an inducible expression plasmid (Fig. 1). This ECD^{HER-2} protein solution was used as the immunogen in mice producing hybridoma clones expressing 5A7 and 11F11 monoclonal antibodies recognizing p185^{HER-2} in ELISA assays, Western immunoblot and immunohistochemistry of tissue sections but not in viable cells (see below). BALB/c mice were also immunized with viable cells overexpressing p185^{HER-2} as well as the recombinant ECD^{HER-2} protein in order to isolate hybridoma clones 8H11 and 10H8.

Both protein and cell ELISA were used as the primary screening methods for detection of antibodies to p185^{HER-2} from hybridoma supernatants. Supernatants positive in either of these screens were further tested for the presence of antibodies which could detect full-length endogenous p185^{HER-2} in frozen breast cancer tissue from a patient with HER-2/*neu* gene overexpression. The monoclonal antibodies from hybridomas which bound p185^{HER-2} in these screens were 5A7, 11F11, 8H11, and 10H8. The hybridoma cells were isolated as monoclonally derived cell lines after cloning twice by limiting dilution. 5A7, 11F11, and 8H11 were of the IgG₁/κ class and isotype. 10H8 was of the IgG_{2a}/κ class and isotype.

The specificity of the monoclonal antibodies was tested in Western blots, immunoprecipitation assays, immunohistochemistry, and immunofluorescence. By ELISA, all four monoclonal antibodies bound to ECD^{HER-2} purified from bacterial cell lysates. Cell ELISA performed with whole cells either overexpressing or not expressing HER-2/*neu* showed that all four antibodies bound p185^{HER-2} on NIH/189 cells but did not bind to antigens on the surface of wild-type NIH 3T3 cells (Table 1). In Western blots, three of the monoclonal antibodies, 5A7, 11F11, and 10H8, recognized human p185^{HER-2} from either SKBR-3 cells (endogenous) or NIH/189 cells (transfected) (Fig. 2). There was no cross-reactivity identified with other proteins in these cell lines or with EGF-R expressed in A431 cells. The 8H11 monoclonal antibody did not detect denatured p185^{HER-2} on Western blots. Both 10H8 and 8H11 were able to bind to soluble p185^{HER-2} in immunoprecipitation reactions while 5A7 and 11F11 were not able to bind to soluble p185^{HER-2} (Fig. 3). The presence of additional bands in the immunoblots reflects either alternatively spliced variants or proteolytically degraded fragments of p185^{HER-2}.

Recognition of endogenous p185^{HER-2} in membranes of human tissue. The ability to bind endogenous forms of p185^{HER-2} in breast cancer tissue was tested with known low- and high-expression tissue samples by immunohistochemistry. All four monoclonal antibodies recognized endogenous p185^{HER-2} on the cell membranes of tumor cells from a frozen human breast cancer biopsy (Table 1). As a control, the monoclonal antibodies were tested on a known low-expressor of p185^{HER-2} and did not show localization in the membranes (data not shown). The utility of these monoclonal antibodies for measuring p185^{HER-2} status in archival paraffin-embedded biopsies was also tested. Similar to the findings in Western blots, 8H11 was unable to recognize p185^{HER-2} while 5A7, 11F11, and 10H8 were able to recognize p185^{HER-2} in the formalin-fixed tissue sections (Fig. 4).

Monoclonal antibody binding to viable intact p185^{HER-2} overexpressing cells. While the utility of these monoclonal antibodies in standard immunoassays was demonstrated, the potential therapeutic utility of these monoclonal antibodies needed to be assessed on viable cells. The binding of monoclonal antibodies to p185^{HER-2} was assessed in viable, intact cells by flow cytometric assays using SKBR-3 human breast cancer cells. Only two monoclonal antibodies, 8H11 and 10H8, were able to bind to p185^{HER-2} expressed on the surface of these cells (Fig. 5). Both 8H11 and 10H8 shifted the population of cells indicating a high level of fluorescence labeling. 5A7 and 11F11 did not bind to SKBR-3 cells and exhibited a fluorescence equivalent to that of control IgG antibody. Similar observations were made by fluorescence microscopy (data not shown).

Internalization and cellular trafficking of anti-p185^{HER-2} antibodies in p185^{HER-2} overexpressing cells. Most targeted therapies require antibodies to be able to target cell-specific antigens and trigger internalization. To determine the subcellular distribution of bound monoclonal antibodies, radioimmunoassays were performed to determine the relative amounts of labeled antibodies in each compartment of the cultured NIH/189 cells. The relative percentage of ¹²⁵I labeled 8H11 and 10H8 in different cellular fractions was found to be similar for both 8H11 and 10H8 (Fig. 6). In these pulse-chase experiments, 8H11 and 10H8 were found to be almost exclusively surface-bound at the beginning of the experiment (T=0 hour), and to be internalized subsequently. Surface label decreased to 35% and 25% of total cpm for 8H11 and 10H8, respectively, over a 24 hour period when maintained at 37°C (Fig. 6A and 6C). At 1 hour of incubation, intracellular ¹²⁵I rose to a peak and then leveled off throughout the assay. The net loss of surface-bound labeled monoclonal antibody was offset by a net increase in supernatant radioactivity. This supernatant fraction was further divided between TCA precipitable and TCA soluble fractions representing intact monoclonal antibody and degraded monoclonal antibody fragments. The net increase of TCA soluble counts rose faster than the TCA precipitable fractions reflecting a higher proportion of internalized, lysosomally degraded, and exocytosed monoclonal antibody fragments compared with passively shed monoclonal antibody (Fig. 6B and 6D). After 24 hours, 45% of monoclonal antibody 8H11 was internalized, degraded, and exocytosed (Fig. 6A). A similar amount of monoclonal antibody 10H8 was internalized but a higher fraction of the monoclonal antibody was shed (Fig. 6C). As a control to test non-specific internalization, labeled 8H11 and 10H8 were both found exclusively in the supernatant fraction of NIH 3T3 cells. Separation of TCA precipitable from TCA soluble fractions showed that both 8H11 and 10H8 were found exclusively as intact proteins (data not shown). Thus, neither 8H11 and 10H8 bound to proteins on the surface of NIH 3T3 cells nor were they internalized.

Isolation and cloning of variable fragments of heavy and light immunoglobulin chains

PCR reactions using degenerate oligonucleotides optimized from the variable regions of mouse heavy and light leader sequences amplified the variable heavy and light chain genes for the

8H11 and 10H8 mAbs (Table 2) (32). After RNA isolation for hybridoma cell lines secreting the 8H11 and 10H8 mAbs, reverse transcription reactions resulted in copying of cDNA. Figure 7 depicts the scheme for isolation and cloning of the VH and VL chain gene of the mAbs. PCR reactions using the VHBi3d and HCRegi2 primers yielded the VH chains of both 8H11 and 10H8 (Figure 8). Likewise, the VkBi7 and LCRegi2 primers yielded the VL chains of both mAbs. PCR products with deoxyadenosine overhangs were ligated into a "TA Cloning" vector plasmid with deoxythymidine overhangs (Invitrogen, Carlsbad, CA). Competent cells were transformed and colonies harboring plasmids with PCR products were selected by blue-white screening according to the manufacturer's protocols. Positive clones were sequenced by the Sanger dideoxy method to confirm that PCR products cloned were indeed bona fide mouse immunoglobulin genes. Using predicted amino acid sequences derived from nucleotide sequences, we confirmed that all four cDNAs were indeed mouse VH and VL genes. Based on Kabat's database of mouse immunoglobulin genes, each variable region belongs to a known family of heavy or light immunoglobulin types (33). VH8H11 belongs to Subgroup IA while VH10H8 belongs to Subgroup IID of the variable region heavy chain sequences. For mouse variable region light chain sequences, VL8H11 matched as a family member of Subgroup IV. VL10H8 matched sequences similar to Subgroup II. In addition, we confirmed that the predicted amino acid sequences were identical to tryptic peptide fragments sequenced by protein mass spectrometry.

Splice overlap extension (SOE) PCR to generate single-chain variable fragments (scFv)

In order to generate antigen-binding protein conformation by apposition of the variable light and variable chains, we used a technique to SOE ("sew") two cDNA fragments together using a novel application of PCR. The scheme for SOE is depicted in Figure 9. In both 8H11 and 10H8, we synthesized primers with overlapping ends that would provide the basis of complementarity between two distinct cDNAs. Tables 3 and 4 list the primers used in two sets of SOE PCR reactions to generate the scFv of 8H11 and 10H8. In fact, three distinct templates were amplified using these extended primers to generate two SOE PCR reactions per mAb. The first template to be used was the anti-CEA mAb heavy chain leader signal peptide and its endogenous Kozak sequence with ribosome binding site. This sequence is necessary for the expression and secretion of the engineered forms of 8H11 and 10H8 in the Glutamine Synthetase Protein Expression system described below.

The first PCR reactions were straightforward amplifications of the three template components needed for eventual fusion into one cDNA. All three templates were amplified using primer pairs A+B, C+D, and E+F. Then the first two templates to be SOEed were the products AB and CD. Under the conditions of overlap extension, a new template was formed from separated sense and antisense strands of both AB and CD which, upon low temperature conditions at 37°C, were allowed to re-anneal at the primer overlap sequences between the B primer and the C primer. Extension using Taq polymerase created a new fusion between AB and CD depicted as AD. Since there is so little product, ethidium bromide staining of agarose electrophoresed PCR products is undetectable. The next step relies on the presence of this AD product for amplification now using primer pairs of A and D. Once enough of this template is amplified, another round of SOE PCR is performed, this time with purified EF template. And finally, amplification by standard PCR of the AF product yields the scFv. The cloned sequences were again ligated into a TA cloning vector. These sequences were checked by automated sequencing for expected splice success and presence of mutations from Taq PCR. In our two cases, there was only a silent mutation in the VL of the 8H11 that had no effect on the serine encoded by either degenerate codon.

Cloning into the minibody expression vector for production of a divalent, engineered antibody

Two forms of the linker between the scFv and the CH3 human constant domain were tested. The Flex linker, which has the human constant hinge domain sequence, is made up of synthetic sequences to add flexibility to the eventual dimer of scFvs formed. The VE form of the minibody is tested to see if a constrained conformation of the divalent scFvs enhances the avidity of the eventual minibody. Instead of 17 amino acids joining scFv to the CH3, the VE minibody only has a linker of valine and glutamic acid residues. Successful in-frame ligation of the 10H8 scFv into both minibody expression vectors was confirmed by automated sequencing.

Key Research Accomplishments:

- produced monoclonal antibodies to the extracellular domain of HER-2/*neu* protein receptor
- characterized antibodies for ability to bind to receptor *in vitro* and *in vivo*
- characterized antibodies for ability to bind living breast cancer cell lines and determine internalization ability
- variable region genes for both heavy and light chain regions for monoclonal antibodies 8H11 and 10H8 were isolated and cloned
- corresponding VL and VH regions were spliced together to form a single chain antibody chimeric gene (scFv)

Reportable Outcomes:

Manuscripts:

1. **Jinha M. Park**, Xiaowei Yang, John J. Park, Oliver W. Press, and Michael F. Press. Assessment of Novel Anti-p185^{HER-2} Monoclonal Antibodies for Internalization-Dependent Therapies. *Hybridoma*, 18(6): 487-495, 1999.

Poster presentation:

1. **Park, J.M.**, Yang, X., Press, O.W., and Press, M.F. "Internalization of Monoclonal Antibodies by HER-2/*neu* Overexpressing Cells." Poster presented at the American Association for Cancer Research 89th Annual Meeting in New Orleans, LA, on March 31, 1998. Abstract in the *Proceedings of the American Association for Cancer Research*, 39: 439, 1998.

Patents and Licenses:

Jinha M. Park and Michael F. Press, Co-Inventors along with the University of Southern California. U.S. Provisional Patent Application. "Variable Region Genes Which Encode Monoclonal Antibodies Triggering Internalization into Cells." Filed on November 15, 1999 with the United States Patent and Trademark Office.

Doctoral Degree Awarded:

Jinha M. Park successfully completed the requirements for the Doctor of Philosophy degree and will be conferred both M.D. and Ph.D. degrees simultaneously in May 2001 at the Keck School of Medicine at the University of Southern California.

Development of Cell Lines and Novel Recombinant Genes:

Four hybridoma cell lines have been developed which secrete monoclonal antibodies against the HER-2/*neu* receptor protein. The genes encoding the variable regions have been cloned from monoclonal antibodies 8H11 and 10H8. They have been sequenced and enjoined as single chain antibody genes.

Personnel Receiving Pay for this Research Effort:

Jinha M. Park, Pre-doctoral Trainee

CONCLUSIONS

Targeted gene therapy and immunotherapy capitalize on the strategies of viruses and biological toxins for cellular internalization after docking to surface receptors. Receptor-mediated endocytosis is the most common mode of vector and toxin internalization (34, 35). Rational cell-specific vector design for gene therapy and immunotherapy thus requires development of monoclonal antibodies directed to cell surface antigens, such as p185^{HER-2}, expressed by target cells (17, 19, 36). Furthermore, the internalization potential of these monoclonal antibodies should be assessed prior to retargeting of gene therapy or immunotherapy vectors to ensure efficient delivery of genes or toxins to subcellular sites of action. Here we used a radioimmunoassay to identify two novel monoclonal antibodies which were internalized after binding a cell surface receptor and, therefore, may be useful for delivery of genes and/or toxins into target cells via an endolysosomal pathway.

Some monoclonal antibodies mimic the effects of ligands in experimental systems. Monoclonal antibodies directed against the rat p185 receptor trigger receptor dimerization, phosphorylation, and downregulation of rat p185, whereas monovalent Fab' have not (37). Another p185^{HER-2} monoclonal antibody was shown to undergo receptor-mediated endocytosis by electron microscopy following capping at membranes of p185^{HER-2} overexpressing NIH 3T3 cells (31). Intracellular trafficking was previously documented by immunogold detection of other monoclonal antibodies in membrane-bound organelles (30). Although this assay has been used to determine the subcellular fate of the murine precursor to the therapeutic monoclonal antibody HerceptinTM, only a few other monoclonal antibodies in experimental protocols for immunotoxin therapies or targeted gene therapies have been tested for internalization (31, 38, 39). Furthermore, the radioimmunoassays used did not identify evidence of endolysosomal trafficking. Here, a rapid radioimmunoassay has been tested for its utility in determining the post-binding subcellular fates of novel monoclonal antibodies to the p185^{HER-2} receptor.

Evidence of 8H11 and 10H8 antibody trafficking through receptor-mediated endocytosis and degradation by lysosomal sorting was demonstrated. TCA soluble fractions of supernatant counts were found in 8H11 and 10H8 assays. No TCA soluble counts were found in the supernatants of NIH 3T3 pulsed control cells. The radioimmunoassay therefore delineated the fates of antibodies to be assessed for receptor-mediated endocytosis.

Future retargeting of vectors and toxins can be improved by characterizing the post-binding fates of the engineered constructs. In particular, non-viral antibody vectors which lack viral endosome escape domains have been fused with viral or toxin polypeptides known to disrupt endosomal membrane integrity (16, 35, 40, 41, 42). Endosomal routing is critical for these added domains to mediate cytoplasmic entry of molecular conjugates. Since divalency is required for antibodies to trigger receptor dimerization and internalization, divalent binding sites may be needed in at least some engineered vectors to ensure receptor-mediated endocytosis (37, 43). Therefore, the radioimmunoassay described may be useful in predetermining antibodies and antibody-based vectors for dimerization and subsequent receptor-mediated endocytosis.

To develop effective monoclonal antibody-based therapies, determining the internalization potential and, particularly, the trafficking of monoclonal antibody-antigen to an endolysosomal pathway is important. After altering targeting specificities, prokaryotic and plant toxin-monoclonal antibody conjugates and targeted viral and non-viral vector conjugates should be assessed for retention of internalization potential. Hence, the radioimmunoassay which determined the internalization of monoclonal antibodies 8H11 and 10H8 could be used to assess the subcellular fates of newly developed monoclonal antibodies against cancer specific surface antigens to improve internalization-dependent therapies.

In this training grant work, the 8H11 and 10H8 antibodies have been described as having internalization potential upon binding HER-2/*neu* overexpressing cells. Further work addressed to the Statement of Work stopped short of retroviral retargeting. The ability to isolate the variable region gene sequences of 8H11 and 10H8 were confirmed by protein microsequencing of tryptic digests of the parental IgG molecules. Both the VH and VL regions of monoclonal antibodies 8H11 and 10H8 were isolated, sequenced, and spliced to form single-chain variable fragments or single-chain antibodies. However, the ability to express and characterize these single-chain antibodies have met with limited success so far. Indeed, the technology to alter retroviral vectors have not dramatically improved since the inception of this grant project. Target specific gene therapy has not met with dramatic improvements in transduction efficiencies. The principal investigator, however, feels that the purposes of training in breast cancer research has been met with unqualified success and every expectation has been fulfilled. The mentor is also very pleased with the results of this grant and the work submitted herein. For the future, the technical difficulties are being addressed with collaborations with experts in single-chain antibody technology and retroviral retargeting. These collaborators are sharing their expertise in continuing this worthwhile project forward as the trainee continues along further training in medicine armed with the experiences of completing his doctoral dissertation.

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APPENDICES

A. Figures

Figure 1. Purification of recombinant HER-2/*neu* extracellular domain (ECD) protein with poly-His tag. The cDNA of HER-2/*neu* was cut between the NcoI and SphI sites and subcloned into an expression vector for production of a 70 kDa epitope-tagged protein. TM=transmembrane domain. Purified protein was resolved by SDS-PAGE, transferred to nitrocellulose and detected by an antibody to the poly-His tag.

Figure 2. Western blots of total protein lysates from human and mouse cell lines. Monoclonal antibodies 5A7, 11F11, 8H11, and 10H8 were tested for binding to p185^{HER-2} in total protein lysates from normal human mammary epithelial cells (HMEC), human breast carcinoma cells overexpressing HER-2/*neu* (SKBR-3), human epidermoid carcinoma cells overexpressing EGF receptor (A431), NIH3T3 cells which lack HER-2/*neu* expression, and NIH/189 cells engineered to overexpress HER-2/*neu*.

Figure 3. Immunoprecipitation of p185^{HER-2} by monoclonal antibody 8H11. Total protein lysates were incubated with the 8H11 monoclonal antibody. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. p185^{HER-2} was detected by a rabbit polyclonal antibody recognizing a carboxy-terminal epitope of p185^{HER-2}.

Figure 4. Immunohistochemical localization of p185^{HER-2}. Tissue sections from a single archival paraffin-embedded breast tumor were analyzed for monoclonal antibody localization to membranes of carcinoma cells. Membrane localization of anti-p185^{HER-2} monoclonal antibodies were detected by the peroxidase-anti-peroxidase method.

Figure 5. Flow cytometry using novel monoclonal antibodies to detect p185^{HER-2} on SKBR-3 cells. SKBR-3 cells overexpressing p185^{HER-2} were incubated with indicated monoclonal antibodies and then tagged with secondary FITC-labeled antibodies. 8H11 and 10H8 monoclonal antibodies detected p185^{HER-2} on the surface of viable SKBR-3 cells. IgG isotype control, 5A7, and 11F11 show no fluorescence.

Figure 6. Receptor-mediated endocytosis assays showing internalization and catabolism of monoclonal antibodies 8H11 and 10H8 by NIH/189 cells. (Top panels) Monoclonal antibodies labeled with ¹²⁵I were incubated on ice with NIH/189 cells. % Total cpm indicate fractions of label on the surface, within the cells, and in the supernatant at various incubation times at 37°C. Mean cpm were calculated for triplicate wells. (Bottom panels) Supernatant cpm was treated with 25% TCA and divided between TCA precipitable cpm (representing intact antibodies) and TCA soluble cpm (representing small MW metabolites of antibodies). Increased TCA soluble cpm fractions shows degradation and exocytosis of internalized monoclonal antibodies. (A and B) 8H11; (C and D) 10H8.

Figure 7. As depicted in the diagram, hybridoma cells were lysed and RNA extracted. RNA was subjected to first strand cDNA synthesis using an oligo-dT primer and MMLV-RT. cDNA was used to isolate the variable heavy and variable light chain gene regions by low stringency conditions of PCR. Optimized degenerate oligonucleotides used to isolate 8H11 and 10H8 genes are listed in Table 1. PCR products were ligated into cloning vectors. Multiple PCR confirmations were performed and several independent clones were manually sequenced to confirm authentic variable mouse IgG genes based on the Kabat database.

Figure 8. Agarose gel electrophoresis of amplified PCR products of VH and VL genes of 8H11 and 10H8 mAbs. Two independent PCR reactions for each set of variable genes were run to confirm the reproducibility of low stringency PCR. All PCR reactions primarily isolated the variable region genes except for VL8H11 which showed an additional band at 900 bp. Specific products were excised from the gels (370 bp products) and purified for ligation into cloning vectors for manual sequencing confirmation.

Figure 9. Splice overlap extension PCR to join antibody gene fragments. Diagram showing the sequence of PCR reactions necessary to fuse three DNA templates together while simultaneously adding intervening artificial peptide sequences (Whitlow 218 scFv linker). Step (1) All three templates are amplified using unique primers with annealable overlapping sequences (see Tables 2 and 3). Step (2) First SOE reaction was the ligation of the anti-CEA signal sequence to the VL of 8H11 and 10H8. Then the newly formed template was itself amplified using primers flanking the 5' of the signal sequence and the 3' end of the VL. Step (3) The SOE is repeated in order to ligate the newly formed signal sequence-VL to the VH gene using the primers to insert the 218 linker between the ligated templates. Again, final amplification of the new template formed the scFv for the antibody.

B. Tables

Table 1. Characteristics of anti-p185^{HER-2} Monoclonal Antibodies

Name of mAb	Antigen	Mouse mAb isotype and subclass	ECD protein ELISA ^a	NIH 3T3 wild-type cell ELISA ^b	NIH/189 cell ELISA ^c	Western blot detection of p185 ^{HER-2}	IP of p185 ^{HER-2}	IHC on frozen biopsies	IHC on paraffin biopsies	Indirect immunofluorescence detection of SKBR-3 cells
5A7	ECD protein	IgG ₁ /κ	+	-	+	+	-	+	+	-
11F11	ECD protein	IgG ₁ /κ	+	-	+	+	-	+	+	-
8H11	Live cells	IgG ₁ /κ	+	-	+	-	+	+	-	+
10H8	Live cells	IgG _{2a} /κ	+	-	+	+	+	+	+	+

^aHER-2/*neu* receptor extracellular domain (ECD) polypeptide; ~70 kDa.

^bNIH 3T3 cells lack p185^{HER-2} expression.

^cNIH/189 cells have p185^{HER-2} overexpression.

Abbreviations used: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; IHC, immunohistochemistry.

Table 2. Degenerate Oligonucleotides Used for Isolation of Variable Heavy and Variable Light Chains of mAb 8H11 and mAb 10H8

Primer	Description	Oligonucleotide Sequence
VHBi3	Heavy chain variable domain	5'-GAGGTGAAGCTGCAGGAGTCAGGACCTAGCCTGGTG-3' (PstI site)
VHBi3c	Heavy chain variable domain	5'-AGGT(C/G)(A/C)AGCTGCAG(C/G)AGTC(A/T)GG-3' (PstI site)
VHBi3d*	Heavy chain variable domain	5'-AGGT(C/G)CAGCTGCAG(C/G)AGTC(A/T)GG-3' (PvuII site)
HCRRegi2*	Gamma chain constant domain	5'-CGGAATTCAGGGGCCAGTGGATAGAC-3' (EcoRI site)
VkBi6	Light chain variable domain	5'-GGTGATATCGTGAT(A/G)AT(C/A)CA(G/A)GATGAACCTCTC-3' (EcoRV site)
VkBi7*	Light chain variable domain	5'-GGTGATATC(A/T)TG(A/C)TGACCCAA(A/T)CTCCACTCTC-3' (EcoRV site)
VkBi8	Light chain variable domain	5'-GGTGATATCGT(G/T)CTCAC(C/T)CA(A/G)TCTCCAGCAAT-3' (EcoRV site)
LCRRegi2*	Kappa chain constant domain	5'-CGGAATTCGGATGGTGGGAAGATGGA-3' (EcoRI site)

* Actual primers used in cloning VH8H11, VH10H8, VL8H11, and VL10H8.

Table 3. Overlapping Oligonucleotides Used for Splicing Together Variable Light and Variable Heavy Chains of mAb 8H11

Primer	Description	Oligonucleotide Sequence
8H11/A (KO255A)	anti-CEA IgG heavy leader with signal peptide, Kozak sequence, and restriction enzyme sites	5'-GCGGAATTCTCTAGAGCGCCACCATGGAGACAGACACTCCTG-3' EcoRI <i>XbaI</i>
<i>Sense</i> 8H11/B	Light chain Framework 1 with 6-mer binding to anti-CEA heavy leader	5'-TAGAGATGTATACATGAGAGTGGAGATTGGGTCATCAAGATATCACCTGT-3' <u>Overlaps with 8H11/C</u>
<i>Antisense</i> 8H11/C	Light chain internal	5'-TCCATGTATACATCTCTAGGAGAGAGTCACTATCACT-3' <u>Overlaps with 8H11/B</u>
<i>Sense</i> 8H11/D	Whitlow 218 linker with 22-mer binding to light chain Framework 4	5'-AGATCCGGGCTTGCCGGATCCAGAGGTGGAGCCTTTGATTTCCAACTTGGTGCCT-3' <u>Overlaps with 8H11/E</u>
<i>Antisense</i> 8H11/E	Whitlow 218 linker with 32-mer binding to heavy chain Framework 1	5'-TCCGGCAAAGCCCGGATCTGGCGAGGGATCCACCAAGGGCGAAGTGCAGCTGCA GGAGTCTGGACCTGAGCT-3' <u>Overlaps with 8H11/D</u>
<i>Sense</i> 8H11/F	Heavy chain Framework 4 with 6xHis tag and restriction enzyme sites	5'-GATCGAATTCAAAGCTTGGCGGCCGCCCGGGTCAGTGATGGTGATGGTGATGAGC EcoRI <i>HindIII</i> <u>NotI</u> SmaI <i>Stop</i>
<i>Antisense</i>		GCTGACAGTGACCAGAGTCCCCTT-3'

Table 4. Overlapping Oligonucleotides Used for Splicing Together Variable Light and Variable Heavy Chains of mAb 10H8

Primer	Description	Oligonucleotide Sequence
10H8/A (KO255A)	anti-CEA IgG heavy leader for signal peptide, Kozak sequence, and restriction enzyme sites	5'-GCGGAATTCTCTAGAGCCGCCACCATGGAGACAGACACTCCTG-3' EcoRI <i>XbaI</i>
<i>Sense</i> 10H8/B	Light chain Framework 1 with 6-mer binding to anti-CEA heavy leader	5'-CAAGACTGACAGGCAGGGAGAGTGAGTTTGGGTCATCAAGATATCACCTGT-3' Overlaps with 10H8/C
<i>Antisense</i> 10H8/C	Light chain internal	5'-TCCCTGCCTGTCAAGTCTTGAGATCAAGCCTCCATCTCT-3'
<i>Sense</i> 10H8/D	Whitlow 218 linker with 24-mer binding to light chain Framework 4	Overlaps with 10H8/B 5'-AGATCCCGGGCTTGCCGGATCCAGAGGTGGAGCCCTTTGATTCCAGCTTGGTGCC TCC-3'
<i>Antisense</i> 10H8/E	Whitlow 218 linker with 24-mer binding to heavy chain Framework 1	Overlaps with 10H8/E 5'-TCCGGCAAGCCCGGATCTGGCGAGGGATCCACCAAGGGCGAAGTCCAGCTGCAGG AGTCTGGG-3'
<i>Sense</i> 10H8/F	Heavy chain Framework 4 with 6xHis tag and restriction enzyme sites	Overlaps with 10H8/D 5'-GATCGAATTCAAAGCTTGGCGGCCGCCCGGGTCAAGTGGTGATGGTGATGTGA EcoRI <i>HindIII</i> <u>NotI</u> SmaI <u>Stop</u> GCTCACGGTGACCGTGGTCCCC-3'
<i>Antisense</i>		

Figure 1.

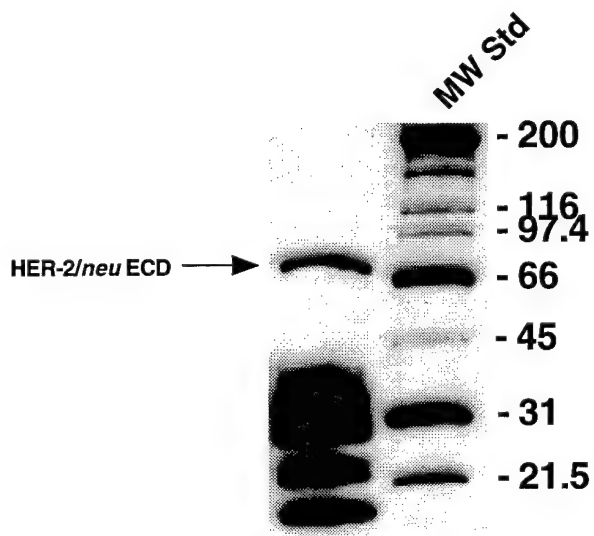
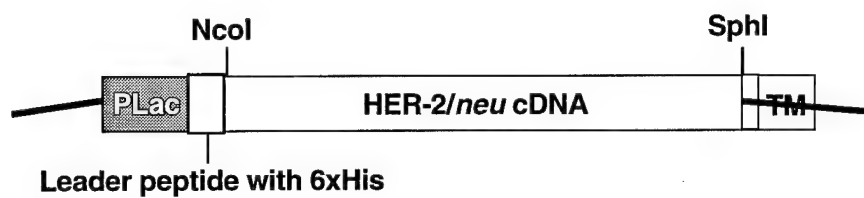


Figure 3.

IP : 8H11 mAb

WB: R60 pAb

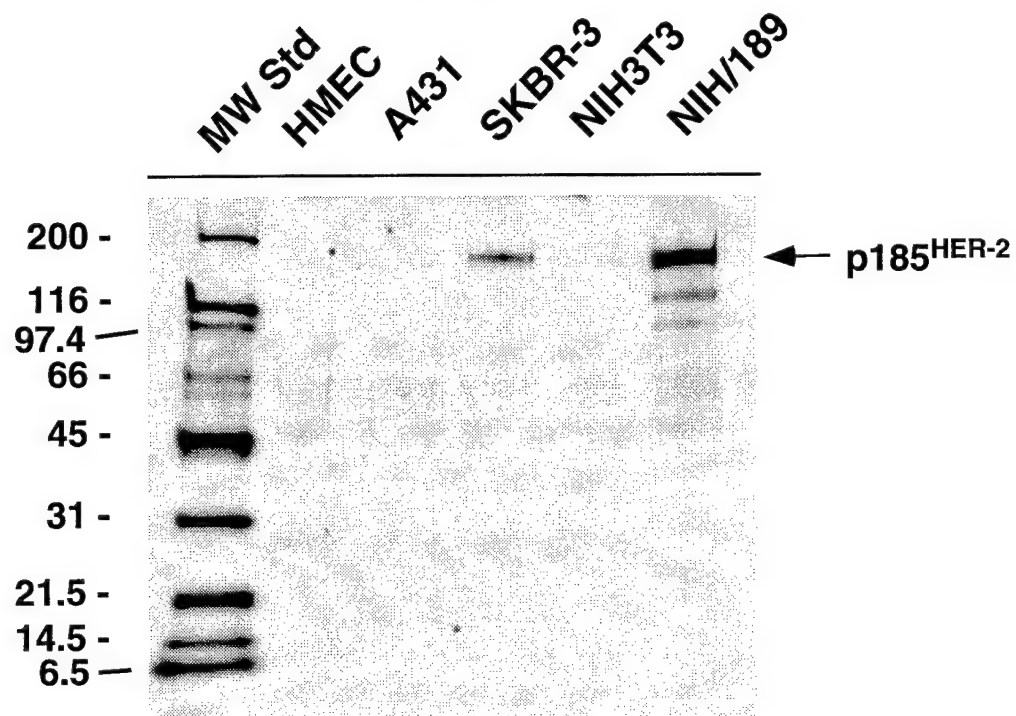


Figure 4.

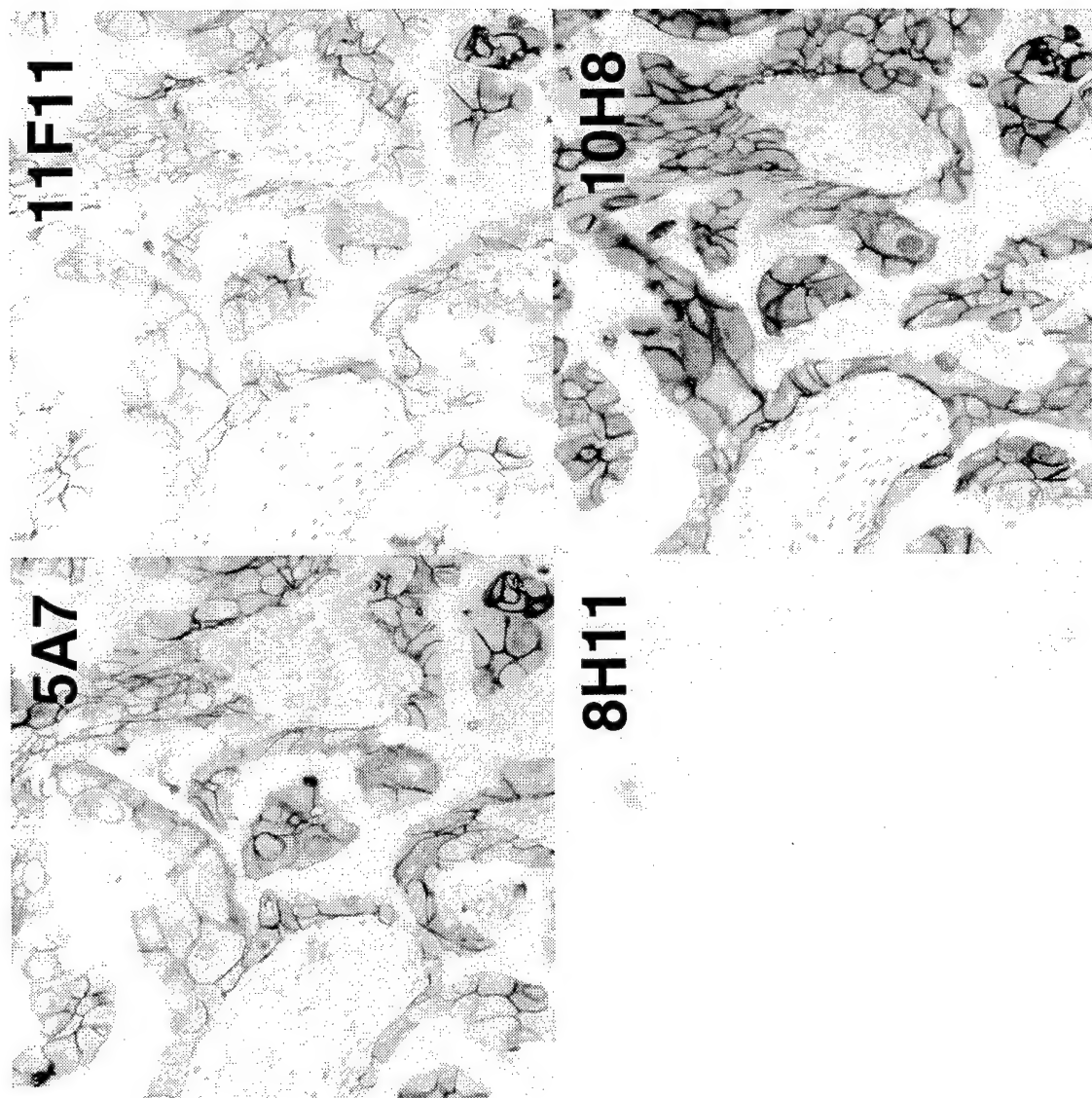


Figure 5.

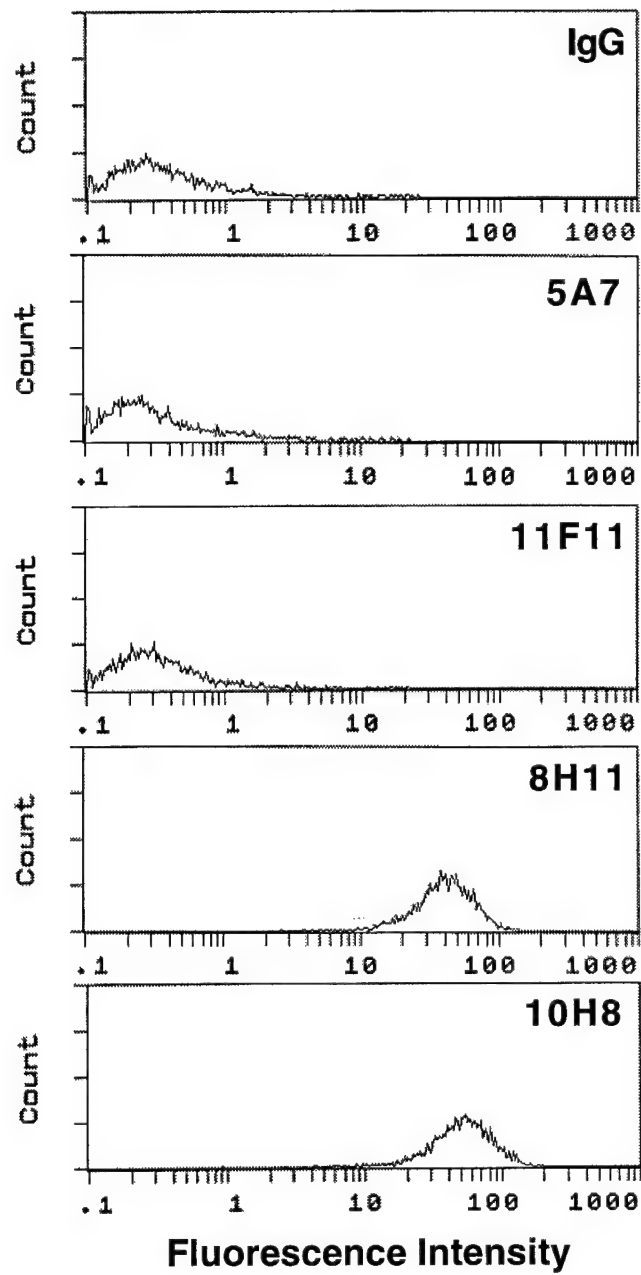
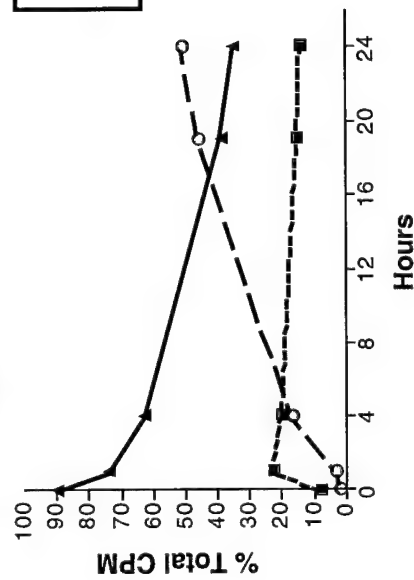
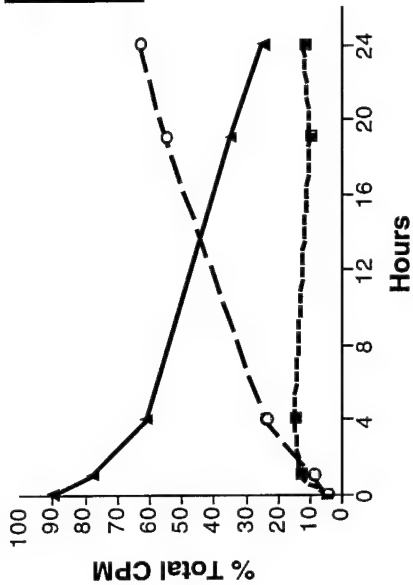


Figure 6.

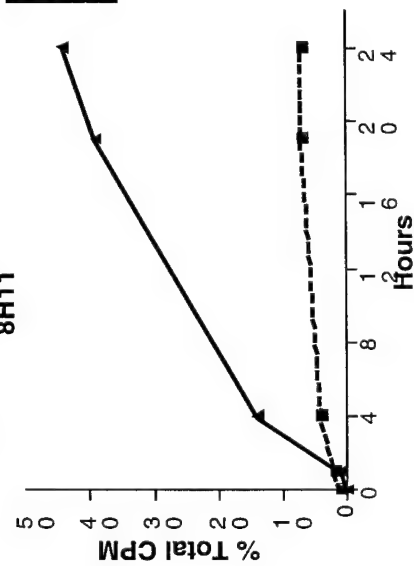
A. mAb 8H11 Internalization and Degradation by NIH/189 Cells



C. mAb 10H8 Internalization and Degradation by NIH/189 Cells



B. NIH/189 Supernatant Fractions of mAb 8H11



D. NIH/189 Supernatant Fractions of 10H8 mAb

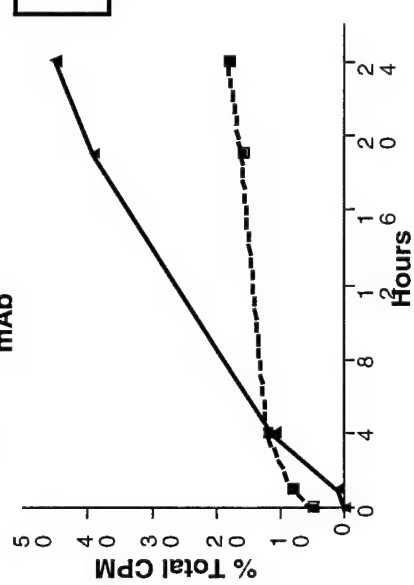


Figure 7. Cloning of Variable Genes from Hybridoma Cells

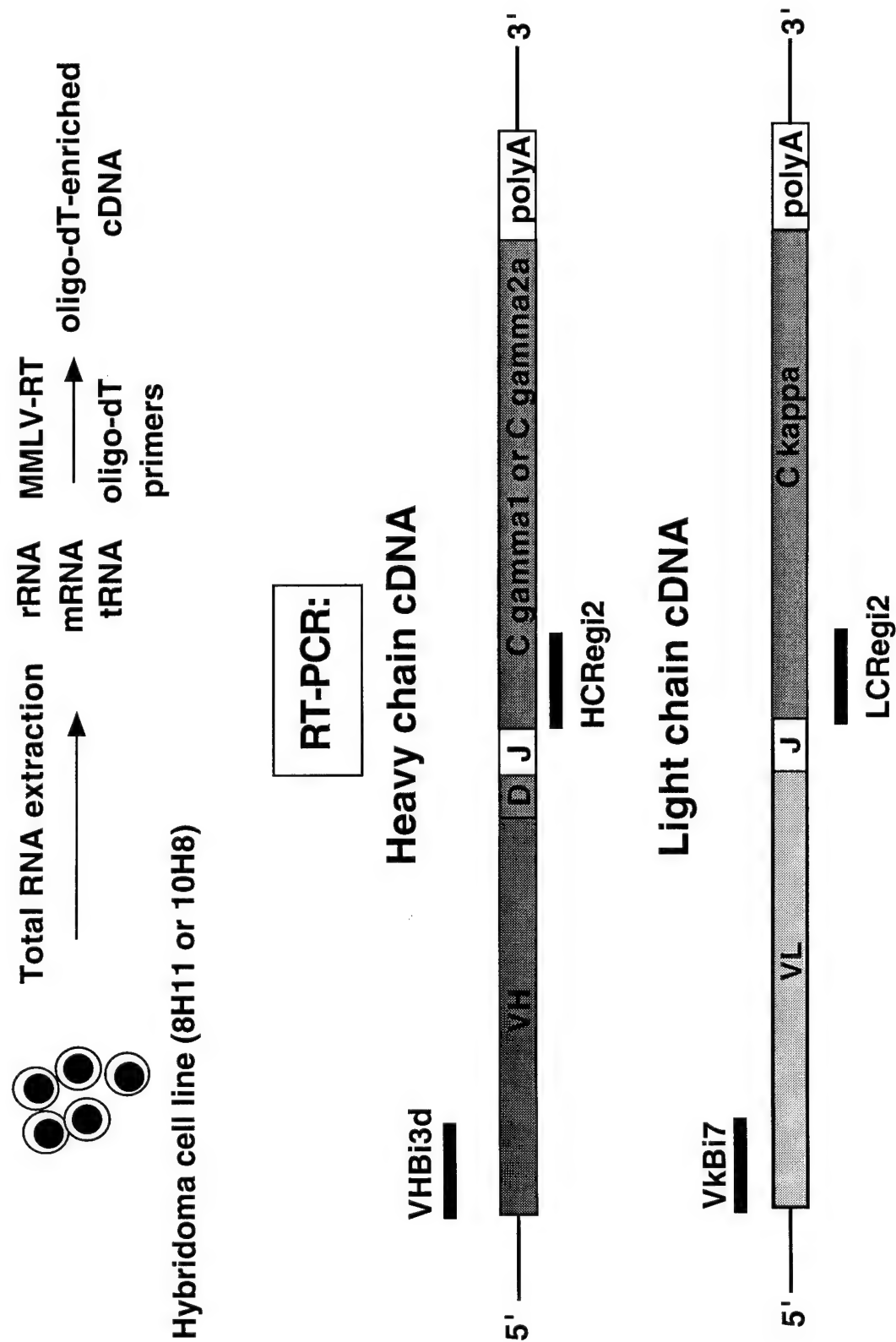


Figure 8.

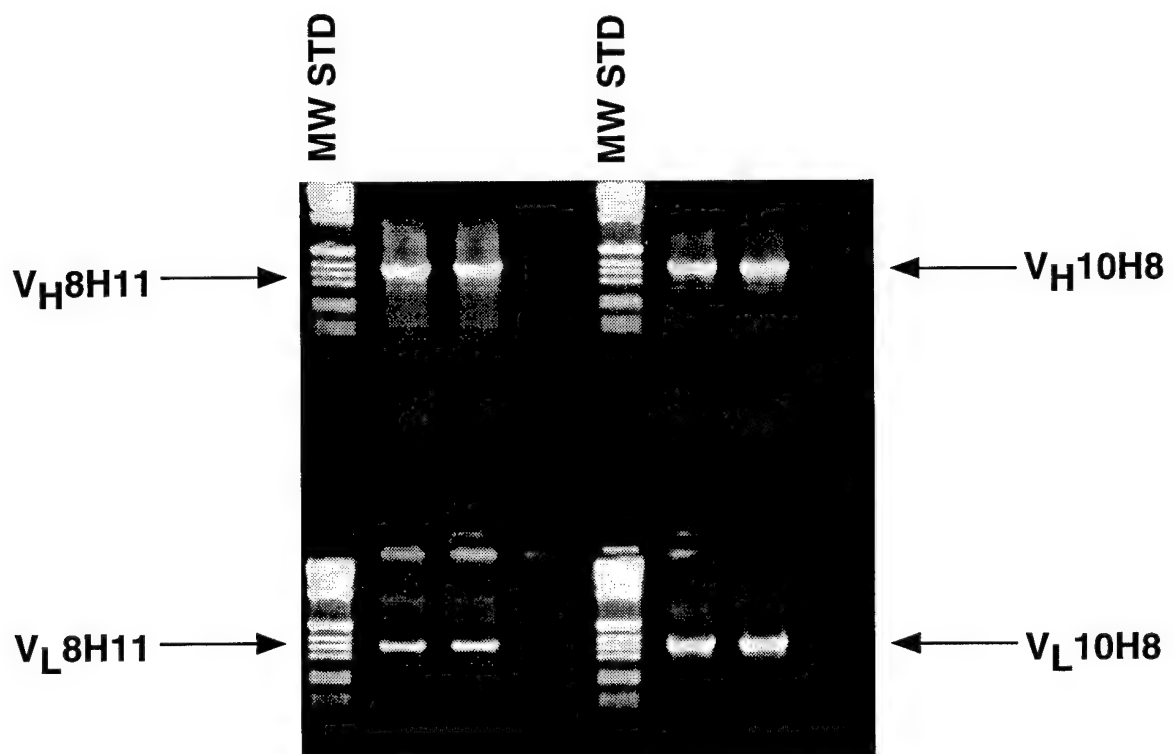
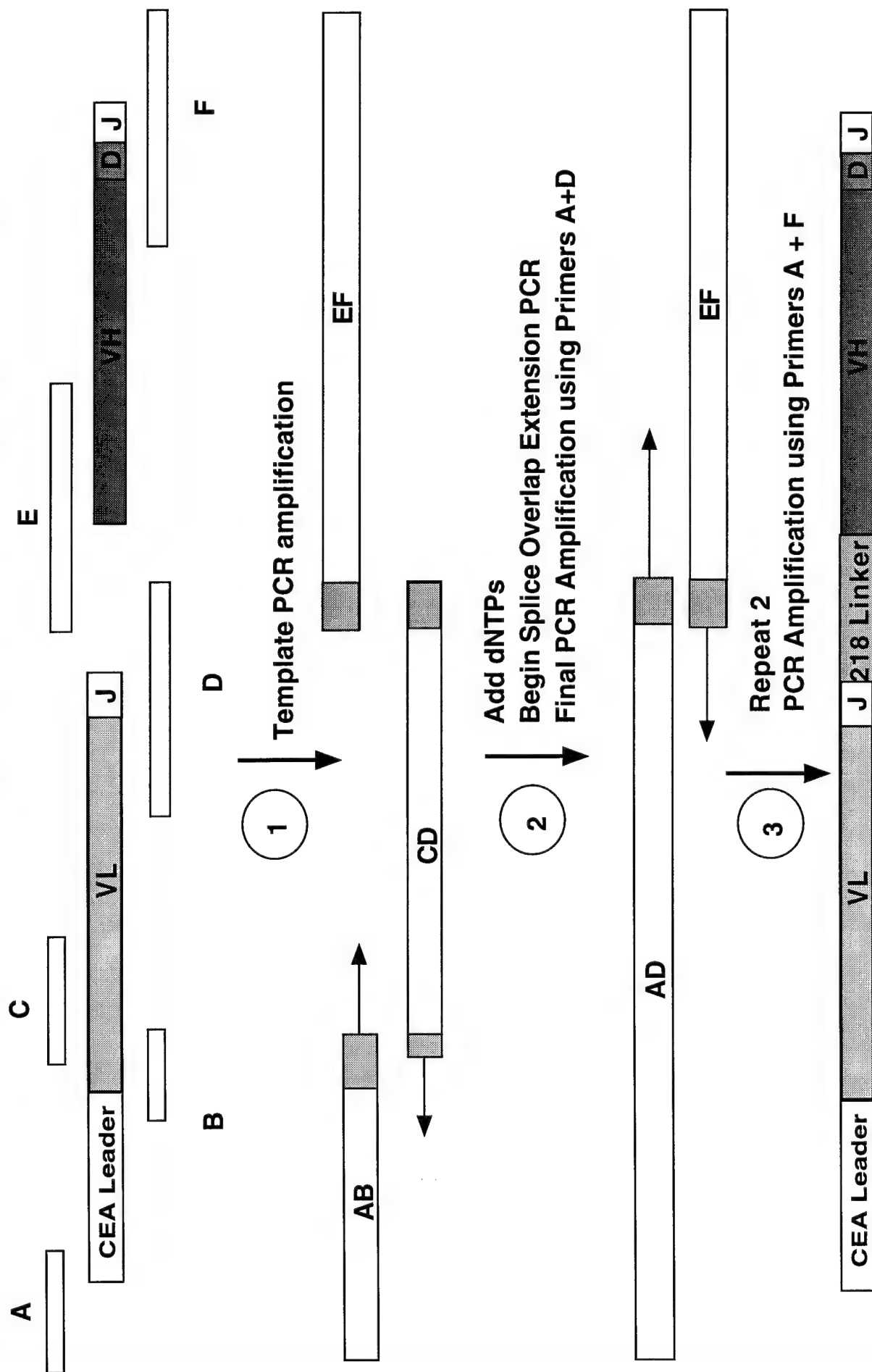


Figure 9. Splice Overlap Extension PCR to Join Antibody Gene Fragments



Assessment of Novel Anti-p185^{HER-2} Monoclonal Antibodies for Internalization-Dependent Therapies

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ABSTRACT

Novel therapies that require internalization of effector domains may be improved by assessing the efficacy of postbinding receptor-mediated endocytosis. To achieve targeted gene therapy of immunotoxin therapy, natural vector-host tropisms must be altered. Recent improvements in monoclonal antibody (MAb) engineering have expanded the potential range of host cells that can be targeted for therapeutic intervention. However, relatively little is known about cellular responses after binding of a vector construct. We have tested the utility of four novel MAbs recognizing the extracellular domain of p185^{HER-2}, a membrane receptor protein, for use in internalization-dependent therapies. All four antibodies bound to p185^{HER-2} in a number of immunoassays. Two antibodies recognized accessible epitopes of p185^{HER-2} on viable cells. Radioimmunoassay demonstrated that antibody-membrane receptor complexes formed by two antibodies were internalized and trafficked through an endolysosomal degradative pathway. Two of the four antibodies evaluated were found to have favorable internalization characteristics suitable for incorporation in a targeting vector. This analytical approach could be applied to antibodies prior to and after fusion with various vectors or toxins to determine the potential utility of the antibodies for targeted therapy.

INTRODUCTION

TARGETED THERAPY for human cancers requires the use of well-characterized reagents that specifically recognize tumor cells in the host background. Recent identification of differences in the genotype and molecular structure of cancer cells has provided an opportunity to develop targetable reagents for the specific therapy of selected human cancers.⁽¹⁻⁴⁾ Amplification of the *HER-2/neu* oncogene in human breast cancers, endometrial cancers, ovarian cancers, gastric cancers, and salivary gland carcinomas provides a potential molecular target for these cancers.⁽⁵⁻⁹⁾ *HER-2/neu* gene amplification is associated with overexpression of the p185^{HER-2} protein. Amplification and overexpression is correlated with a shorter time to relapse and a shorter overall patient survival.^(8,9) A humanized monoclonal antibody (MAb), HerceptinTM (Genentech, South San Francisco, CA), has been used to successfully treat some women with *HER-2/neu* overexpressing breast and ovarian cancers, demonstrating that p185^{HER-2} can be used to selectively target human cancers.^(10,11) While outcomes have improved for pa-

tients treated with HerceptinTM, more effective classes of antibody-based therapies need to be developed to capitalize on cancer specific antigen expression.

Several groups have tested p185^{HER-2} overexpressing cells as a target for gene therapy.⁽¹²⁻¹⁵⁾ Targeted delivery of genes has been attempted by altering cognate receptors on virions with cellular receptor ligands and single-chain antibodies.⁽¹⁶⁻¹⁹⁾ Although these vectors have successfully bound p185^{HER-2} overexpressing cells, none have resulted in significant rates of gene transduction.

Because the successful gene therapy vector will require not only cell targeting, but internalization of the vector and expression of a therapeutic gene, it is important to evaluate as many of these steps as possible prior to construction of the vector. To this end, we isolated several anti-p185^{HER-2} MAbs and characterized their internalization potential using a rapid, quantifiable radioimmunoassay. Two of the four p185^{HER-2} MAbs bound to viable, intact target cells, were internalized and then trafficked through an endolysosomal pathway. These antibodies are expected to be useful in targeting treatment vectors that require internalization for therapeutic effect.

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MATERIALS AND METHODS

Production and isolation of MAbs to the extracellular domain (ECD) of p185^{HER-2} involved the use of recombinant ECD^{HER-2} protein or viable HER-2/*neu* overexpressing cell lines for immunization of BALB/c mice, and screening of hybridomas with enzyme-linked immunoadsorbent assay (ELISA) and immunocytochemical assays. The MAbs were purified by affinity chromatography. The specificity of the antibodies was determined with Western immunoblot analyses, immunoprecipitation assays, and immunohistochemistry. The ability of MAbs to specifically recognize viable HER-2/*neu* overexpressing cell lines was evaluated with fluorescence microscopy and flow cytometry. Cellular trafficking of MAbs was characterized with radioimmunoassay methods.

Protein production and purification

A truncated p185^{HER-2} protein was synthesized and injected into mice as the initial immunogen for generating anti-p185^{HER-2} MAbs. The cDNA of the HER-2/*neu* gene was cut between the NcoI and SphI sites, yielding a portion of the open reading frame, which includes the initiation codon extending to the transmembrane domain.⁽²⁰⁾ The 2-kilobase DNA insert was ligated into the multiple cloning site immediately downstream of an inducible *trp-lac* promoter of a pTrcHisA expression vector (Invitrogen, San Diego, CA). Successful in-frame subcloning of the HER-2/*neu* gene was confirmed by restriction fragment analysis and direct DNA sequencing (Amersham Pharmacia Biotech, Piscataway, NJ). TOP10 *E. coli* (Invitrogen) were transformed using the HER-2/*neu* expression vector and induced to produce a fusion protein containing a leader peptide with six contiguous histidine residues followed by the ECD^{HER-2}. Proteins were purified by conditions using metal chelate affinity chromatography on Ni²⁺-NTA-agarose as suggested by the manufacturer (QIAGEN, Valencia, CA).

Cell culture

In addition to recombinant ECD^{HER-2} protein, p185^{HER-2} overexpressing intact cells (NIH/189 and SKBR-3 cells) were used to immunize BALB/c mice. NIH/189 and NIH 3T3 cells were used as sources of intact or solubilized p185^{HER-2} protein for immunological assays. The NIH/189 cell line, which has been described previously as a p185^{HER-2} overexpressor, was a generous gift from C. Richter King.⁽²¹⁾

NIH 3T3, NIH/189, and A431 human epidermoid carcinoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies). The SKBR-3 human breast adenocarcinoma cell line was grown in McCoy's 5A media supplemented with 10% FBS and penicillin/streptomycin. Normal human mammary epithelial cells (HMEC) were grown in media formulated by the supplier of the cells (Clonetics, San Diego, CA). These cells were used as non-p185^{HER-2} expressing controls for Western blots and immunoprecipitations. Sp2/0-Ag14 mouse myeloma cells were grown in RPMI-1640 medium supplemented with sodium pyruvate, L-glutamine, penicillin/streptomycin, and 15% FBS prior to cell fusion.

Immunization

Two sets of 8- to 9-week-old female BALB/c mice were immunized with either protein or live cells as immunogens. Three female BALB/c mice received three sequential immunizations of 100, 50, and 50 μ g each of the truncated HER-2/*neu* protein. The first immunization of ECD^{HER-2} was an intraperitoneal injection of protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, MO). At weeks 3 and 5, mice were given two immunizations of 50 μ g of protein mixed with Freund's incomplete adjuvant (Sigma). The mouse that yielded two MAbs (5A7 and 11F11) in this work was given three further boosts of 50 μ g ECD^{HER-2}.

Mice were also inoculated with live p185^{HER-2}-overexpressing cells to ensure the widest range of MAbs against all potential ECD^{HER-2} epitopes, including glycosylated regions. Initial immunizations were intraperitoneal injections of $2-5 \times 10^6$ SKBR-3 human breast cancer cells in serum-free RPMI-1640 media over a 7-month period. The mouse that yielded MAbs 8H11 and 10H8 had another boost of 5×10^6 NIH/189 cells in Freund's incomplete adjuvant injected intraperitoneally and 1×10^6 cells injected subcutaneously. The final immunizations before fusion were an intraperitoneal boost of 10×10^6 NIH/189 cells in phosphate-buffered saline (PBS) and an intravenous injection of 50 μ g of ECD^{HER-2} protein in a buffer containing 0.1 M NaH₂PO₄ and 10 mM TrisHCl, pH 8.0.

Hybridoma production and monoclonal antibody screening

Spleen cells from BALB/c mice immunized with either protein or cells were fused to Sp2/0-Ag14 mouse myeloma cells to generate hybridoma cells.⁽²²⁾ Cells were fused with polyethylene glycol in a method modified from Köhler and Milstein.⁽²³⁾ Hybridoma cells were selected for anti-p185^{HER-2} antibody production by ELISA and immunohistochemistry.

Protein and cell ELISA

ELISA was used as the primary method to screen hybridoma cell clone supernatants for secretion of antibodies. ECD^{HER-2} (250 ng/well) protein was coated onto 96-well plates in PBS. After blocking in 3% bovine serum albumin (BSA), 50 μ L of supernatant were incubated from 1–2 h at room temperature. After washing three times with PBS, a 1:2000 dilution (in 3% BSA) of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) was incubated for 30 min at room temperature. Positive wells were visualized by *o*-phenylene diamine (OPD) substrate (Sigma) and read at optical density (O.D.) 490 nm on an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

To ensure detection of antibodies recognizing conformational and polysaccharide epitopes, cell ELISA using NIH3T3 and NIH/189 cells was used to detect such clones. Cells (1×10^4 /well) were fixed using 100% methanol onto 96-well Linbro Titertek (ICN, Irvine, CA) plates after overnight attachment onto 1.5% gelatin (Difco, Detroit, MI). The protocol for protein ELISA was used for cell ELISA.

Immunohistochemistry

Frozen and paraffin-embedded surgical biopsies of both known low and high p185^{HER-2} expressor breast cancers were

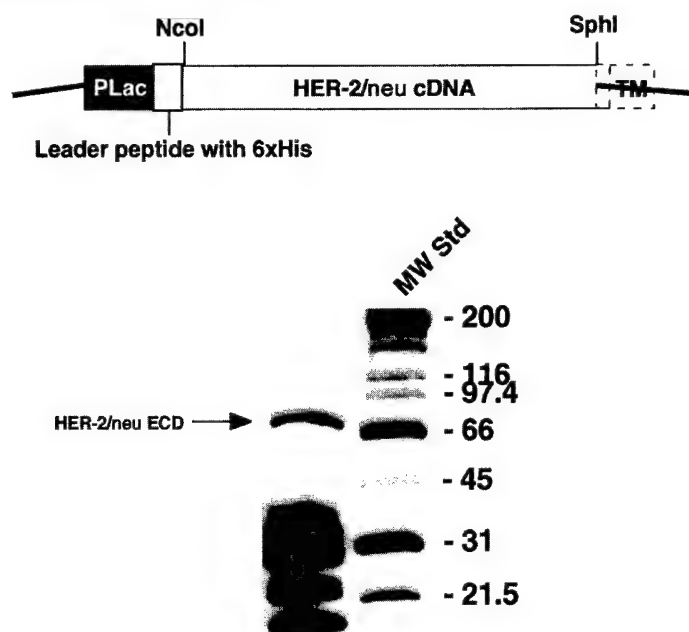


FIG. 1. Purification of recombinant HER-2/neu extracellular domain (ECD) protein with poly-His tag. The cDNA of HER-2/neu was cut between the NcoI and SphI sites and subcloned into an expression vector for production of a 70-kDa epitope-tagged protein. TM = transmembrane domain. Purified protein was resolved by SDS-PAGE, transferred to nitrocellulose and detected by an antibody to the poly-His tag.

tested for binding of p185^{HER-2} by these four MAb. The use of human tissue was approved prior to this work by the Institutional Review Board of the USC School of Medicine.

Frozen tissue section immunohistochemistry was used as a secondary screening method to detect positive hybridomas that secreted antibodies to intact p185^{HER-2} from human tissue. p185^{HER-2} in tissue sections were detected by the peroxidase-anti-peroxidase technique, as previously described.⁽²⁴⁾

Breast cancer cases embedded in paraffin as a single multi-tumor block were also tested for p185^{HER-2} recognition. Formalin-fixed breast cancer biopsies with known low and high expression of p185^{HER-2} protein were embedded in a specified matrix in a single paraffin block.⁽²⁵⁾ MAbs purified from as-

cites were used at equal concentrations to compare localization in a single specimen.

Western blots and immunoprecipitation

MAbs were used to probe for full-length p185^{HER-2} in total protein lysates of mouse and human cell lines loaded equally in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method used has been previously described.⁽²⁶⁾

Immunoprecipitations demonstrated the ability of MAbs to bind to full length HER-2/neu in soluble detergent lysates of mouse and human cell lines. The immunoprecipitation method

TABLE 1. CHARACTERISTICS OF ANTI-p185^{HER-2} MONOCLONAL ANTIBODIES

Name of MAb	Antigen	Mouse mAb isotype and subclass	ECD protein ELISA ^a	NIH 3T3 wild-type cell ELISA ^b	NIH/189 cell ELISA ^c	Western blot detection of p185 ^{HER-2}	IP of p185 ^{HER-2}	IHC on frozen biopsies	IHC on paraffin biopsies	Indirect immunofluorescence detection of SKBR-3 cells
5A7	ECD protein	IgG ₁ /κ	+	—	+	+	—	+	+	—
11F11	ECD protein	IgG ₁ /κ	+	—	+	+	—	+	+	—
8H11	Live cells	IgG ₁ /κ	+	—	+	—	+	+	—	+
10H8	Live cells	IgG _{2a} /κ	+	—	+	+	+	+	+	+

^aHER-2/neu receptor extracellular domain (ECD) polypeptide; ~70 kDa; ^bNIH 3T3 cells lack p185^{HER-2} overexpression; and ^cNIH/189 cells have p185^{HER-2} overexpression.

Abbreviations used: MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; IHC, immunohistochemistry.

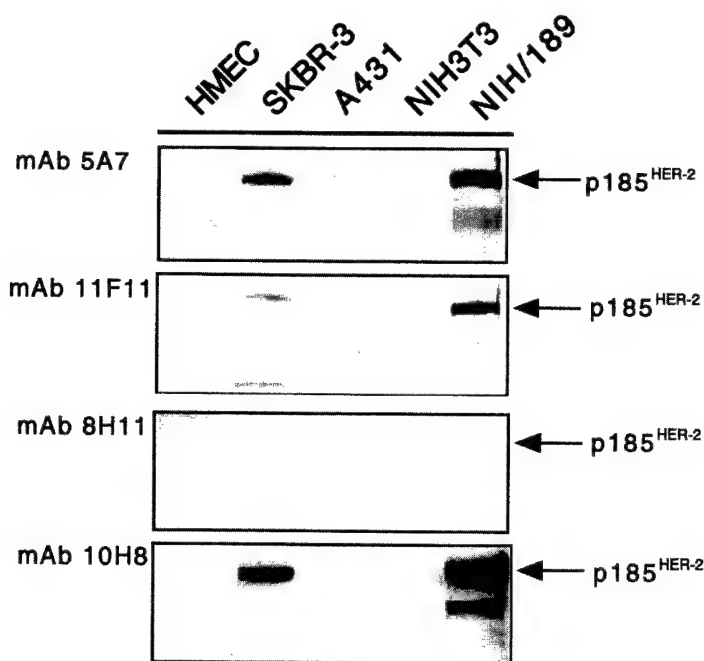


FIG. 2. Western blots of total protein lysates from human and mouse cell lines. MAbs 5A7, 11F11, 8H11, and 10H8 were tested for binding to p185^{HER-2} in total protein lysates from normal human mammary epithelial cells (HMEC), human breast carcinoma cells overexpressing HER-2/*neu* (SKBR-3), human epidermoid carcinoma cells overexpressing EGF receptor (A431), NIH3T3 cells which lack HER-2/*neu* expression, and NIH/189 cells engineered to overexpress HER-2/*neu*.

used has been previously described.⁽²⁷⁾ A rabbit polyclonal antibody (R60) previously reported for use in p185^{HER-2} Western blots was used.⁽⁹⁾ A 1:2000 dilution of R60 polyclonal sera in 10% goat serum was used to detect the C-terminus of immunoprecipitated p185^{HER-2} protein in the blot.

Flow cytometry

A prerequisite for the determination of cellular trafficking of MAb-antigen complexes is the binding of MAb to its target on the surface of p185^{HER-2} overexpressing cells. Fluorescence-activated cell sorting (FACS) was used to determine ligation of MAbs to native p185^{HER-2} on the surface of SKBR-3 cells. The flow cytometry method has been previously described.⁽²⁸⁾ Cells were analyzed on a Coulter Elite ESP Cell Sorter (Beckman Coulter, Miami Lakes, FL). Mouse IgG isotype control was used as a negative control for FACS (Zymed).

Radioimmunoassay

A method to detect internalization and intracellular trafficking of MAbs directed against cell-surface antigens was used as previously described to characterize anti-p185^{HER-2} MAbs with this potential.^(29,30) MAbs 8H11 and 10H8 were labeled with ¹²⁵I (Amersham Pharmacia) using the Iodo-Gen method (Pierce). NIH 3T3 and NIH/189 cells were pulsed with labeled MAbs and chased with 1% BSA/RPMI-1640. Supernatant, acid-releasable surface-bound, and intracellular label were collected and counted on a Cobra Automated Gamma Counter (Packard, Meriden, CT). The supernatant fraction was further divided between 25% TCA precipitable and 25% TCA soluble components representing shed, intact MAbs and degraded MAbs, respectively. These fractions were collected at times of

0, 1, 4, 19, and 24 h. All timepoint determinations were corrected for nonspecific initial binding ($T = 0$ h) by subtracting counts in fractions after surface blocking by unlabeled MAbs.

RESULTS

Four MAbs have been isolated and characterized for their individual characteristics in standard immunoassays and in an internalization assay to determine their potential for use with internalization-dependent therapies.

Production and characterization of MAbs

A 70-kDa, truncated ECD^{HER-2} protein was isolated from inclusion bodies of bacteria which had been transformed by an inducible expression plasmid (Fig. 1). This ECD^{HER-2} protein solution was used as the immunogen in mice producing hybridoma clones 5A7 and 11F11. These MAbs recognized p185^{HER-2} in ELISA assays, Western immunoblot and immunohistochemistry of tissue sections but not in viable cells (see below). BALB/c mice were also immunized with viable cells overexpressing p185^{HER-2} as well as the recombinant ECD^{HER-2} protein to isolate hybridoma clones 8H11 and 10H8.

Protein and cell ELISA were used as the primary screening methods for detection of antibodies to p185^{HER-2} from hybridoma supernatants. Supernatants positive in either of these screens were further tested for the presence of antibodies that could detect full-length endogenous p185^{HER-2} in frozen breast cancer tissue from a patient with HER-2/*neu* gene overexpression. The MAbs from hybridomas that bound p185^{HER-2} in these screens were 5A7, 11F11, 8H11, and 10H8. The hybridoma cells were isolated as monoclonally derived cell lines after

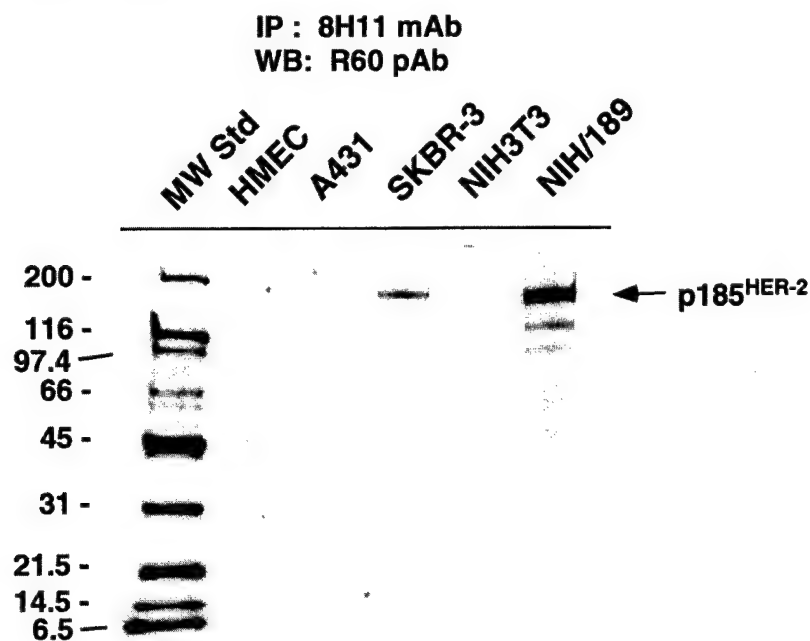


FIG. 3. Immunoprecipitation of p185^{HER-2} by monoclonal antibody 8H11. Total protein lysates were incubated with the 8H11 MAb. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. p185^{HER-2} was detected by a rabbit polyclonal antibody recognizing a carboxy-terminal epitope of p185^{HER-2}.

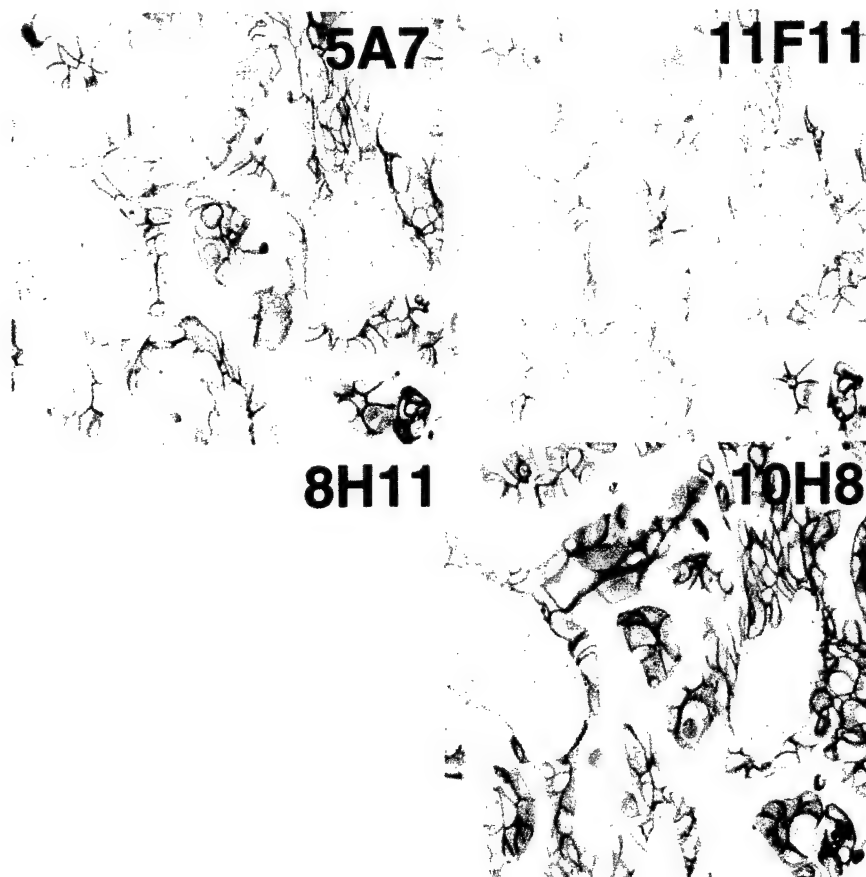


FIG. 4. Immunohistochemical localization of p185^{HER-2}. Tissue sections from a single archival paraffin-embedded breast tumor were analyzed for MAb localization to membranes of carcinoma cells. Membrane localization of anti-p185^{HER-2} MABs were detected by the peroxidase-anti-peroxidase method.

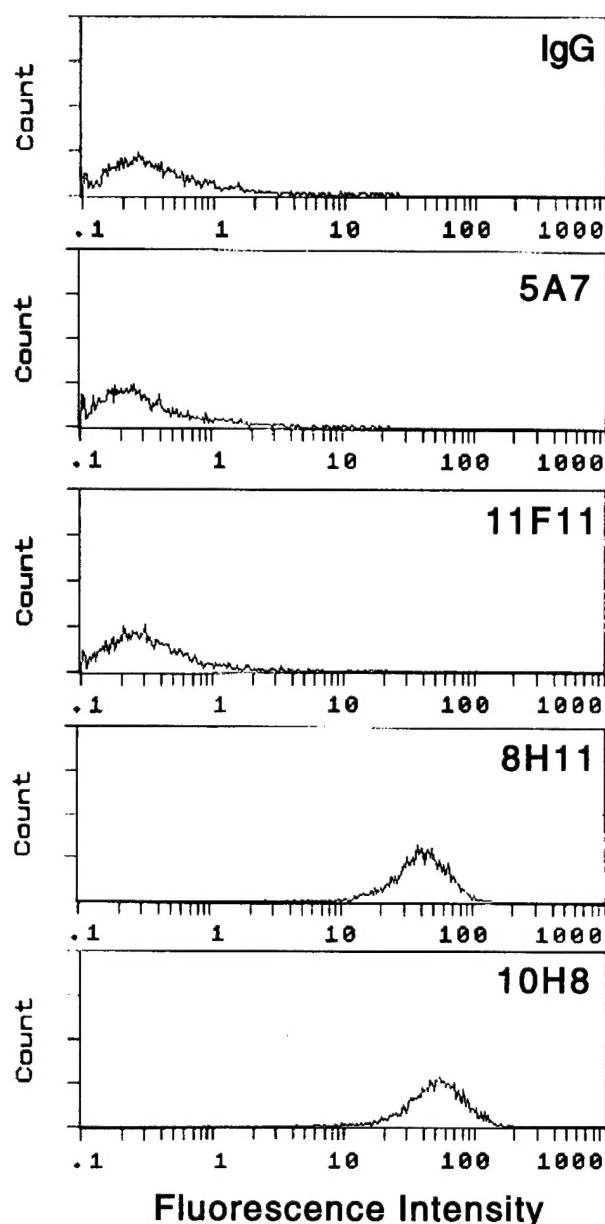


FIG. 5. Flow cytometry using novel MABs to detect p185^{HER-2} on SKBR-3 cells. SKBR-3 cells overexpressing p185^{HER-2} were incubated with indicated MABs and then tagged with secondary FITC-labeled antibodies. 8H11 and 10H8 MABs detected p185^{HER-2} on the surface of viable SKBR-3 cells. IgG isotype control, 5A7, and 11F11 show no fluorescence.

cloning twice by limiting dilution. 5A7, 11F11, and 8H11 were of the IgG₁/κ class and isotype. 10H8 was of the IgG₂/κ class and isotype.

The specificity of the MABs was tested in Western blots, immunoprecipitation assays, immunohistochemistry, and immunofluorescence. By ELISA, all four MABs bound to ECD^{HER-2} purified from bacterial cell lysates. Cell ELISA performed with whole cells either overexpressing or not expressing HER-2/*neu* showed that all four antibodies bound p185^{HER-2} on NIH/189 cells, but did not bind to antigens on the surface of wild-type

NIH 3T3 cells (Table 1). In Western blots, three of the MABs, 5A7, 11F11, and 10H8, recognized human p185^{HER-2} from either SKBR-3 cells (endogenous) or NIH/189 cells (transfected) (Fig. 2). There was no cross-reactivity identified with other proteins in these cell lines or with EGF-R expressed in A431 cells. The 8H11 MAB did not detect denatured p185^{HER-2} on Western blots. Both 10H8 and 8H11 were able to bind to soluble p185^{HER-2} in immunoprecipitation reactions, while 5A7 and 11F11 were not able to bind to soluble p185^{HER-2} (Fig. 3). The presence of additional bands in the immunoblots reflects either alternatively spliced variants or proteolytically degraded fragments of p185^{HER-2}.

Recognition of endogenous p185^{HER-2} in membranes of human tissue

The ability to bind endogenous forms of p185^{HER-2} in breast cancer tissue was tested with known low- and high-expression tissue samples by immunohistochemistry. All four MABs recognized endogenous p185^{HER-2} on the cell membranes of tumor cells from a frozen human breast cancer biopsy (Table 1). As a control, the MABs were tested on a known low-expressor of p185^{HER-2} and did not show localization in the membranes (data not shown). The utility of these monoclonal antibodies for measuring p185^{HER-2} status in archival paraffin-embedded biopsies was also tested. Similar to the findings in Western blots, 8H11 was unable to recognize p185^{HER-2} while 5A7, 11F11, and 10H8 were able to recognize p185^{HER-2} in the formalin-fixed paraffin-embedded tissue sections (Fig. 4).

MAB binding to viable intact p185^{HER-2} overexpressing cells

While the utility of these MABs in standard immunoassays was demonstrated, the potential therapeutic utility of these MABs needed to be assessed on viable cells. The binding of MABs to p185^{HER-2} was assessed in viable, intact cells by flow cytometric assays using SKBR-3 human breast cancer cells. Only two MABs, 8H11 and 10H8, were able to bind to p185^{HER-2} expressed on the surface of these cells (Fig. 5). Both 8H11 and 10H8 shifted the population of cells indicating a high level of fluorescence labeling. 5A7 and 11F11 did not bind to SKBR-3 cells and exhibited a fluorescent distribution equivalent to that of control IgG antibody. Similar observations were made by fluorescence microscopy (data not shown).

Internalization and cellular trafficking of anti-p185^{HER-2} antibodies in p185^{HER-2} overexpressing cells

Most targeted therapies require antibodies to be able to target cell-specific antigens and trigger internalization. To determine the subcellular distribution of bound MABs, radioimmunoassays were performed to determine the relative amounts of labeled antibodies in each compartment of the cultured NIH/189 cells. The relative percentage of ¹²⁵I labeled 8H11 and 10H8 in different cellular fractions was found to be similar for 8H11 and 10H8 (Fig. 6). In these pulse-chase experiments, 8H11 and 10H8 were found to be almost exclusively surface-bound at the beginning of the experiment (*T* = 0 h), and to be internalized subsequently. Surface label decreased to 35% and 25% of total cpm for 8H11

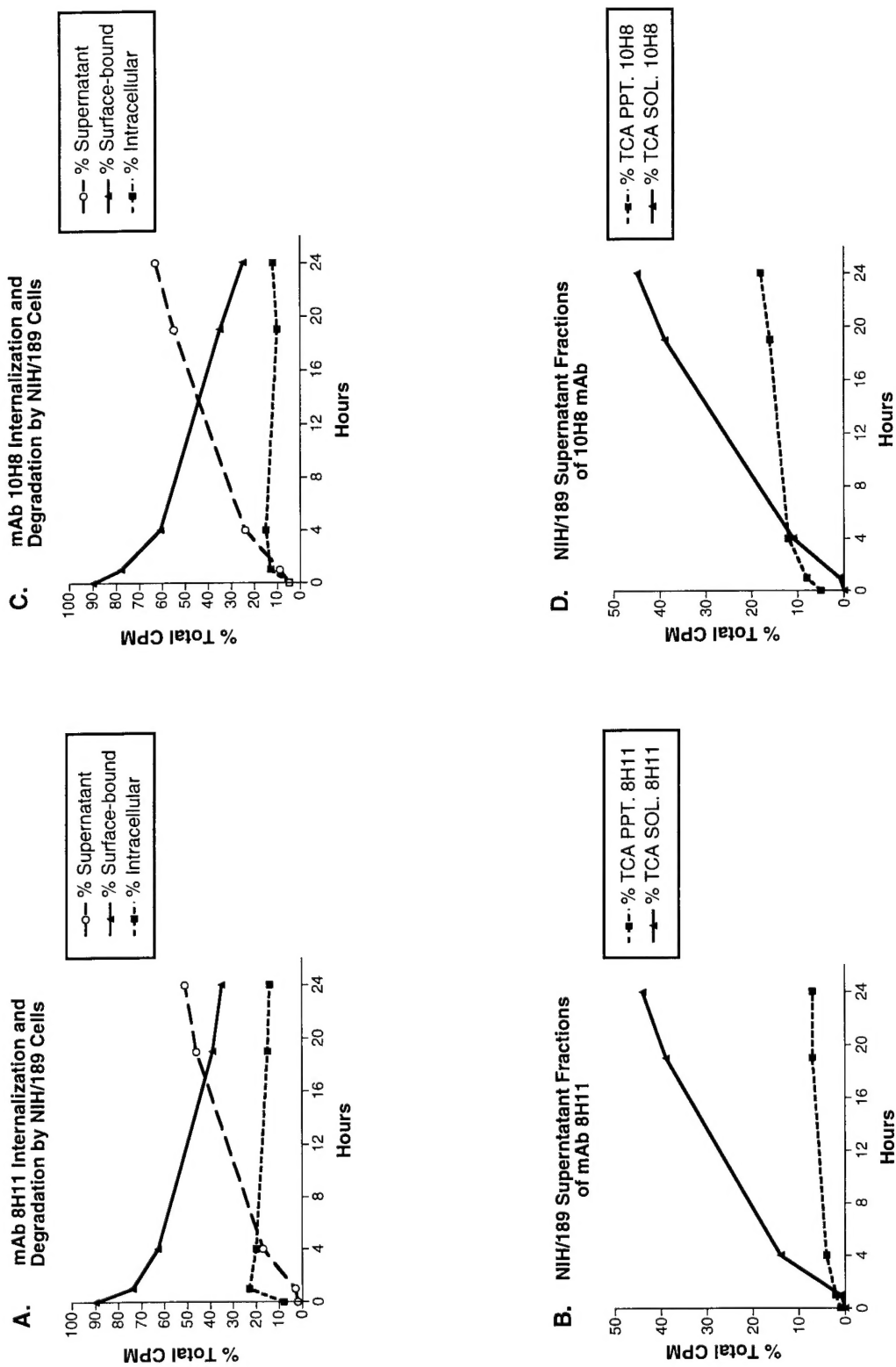


FIG. 6. Receptor-mediated endocytosis assays showing internalization and catabolism of monoclonal antibodies 8H11 and 10H8 by NIH/189 cells. MAb (top panels) labeled with ^{125}I were incubated on ice with NIH/189 cells. Percent total cpm indicate fractions of label on the surface, within the cells, and in the supernatant at various incubation times at 37°C . Mean cpm were calculated for triplicate wells. (Bottom panels) Supernatant cpm was treated with 25% TCA and divided between TCA-precipitable cpm (representing intact antibodies) and TCA-soluble cpm (representing small MW metabolites of antibodies). Increased TCA-soluble cpm fractions shows degradation and exocytosis of internalized MAb. (A and B) 8H11; (C and D) 10H8.

and 10H8, respectively, over a 24-h period when maintained at 37°C (Fig. 6A and 6C). At 1 h of incubation, intracellular ^{125}I rose to a peak and then leveled off throughout the assay. The net loss of surface-bound labeled MAb was offset by a net increase in supernatant radioactivity. This supernatant fraction was further divided between TCA-precipitable and TCA-soluble fractions representing intact MAb and degraded MAb fragments. The net increase of TCA-soluble counts rose faster than the TCA-precipitable fractions reflecting a higher proportion of internalized, lysosomally degraded, and exocytosed MAb fragments compared with passively shed MAb (Fig. 6B and 6D). After 24 h, 45% of MAb 8H11 was internalized, degraded, and exocytosed (Fig. 6A). A similar amount of monoclonal antibody 10H8 was internalized but a higher fraction of the monoclonal antibody was shed (Fig. 6C). As a control to test nonspecific internalization, labeled 8H11 and 10H8 were found exclusively in the supernatant fraction of NIH 3T3 cells. Separation of TCA-precipitable from TCA-soluble fractions showed that 8H11 and 10H8 were found exclusively as intact proteins (data not shown). Thus, 8H11 and 10H8 neither bound to proteins on the surface of NIH 3T3 cells nor were internalized by endocytosis, trafficked to lysosomes, and exocytosed.

DISCUSSION

Targeted gene therapy and immunotherapy capitalize on the strategies of viruses and biological toxins for cellular internalization after docking to surface receptors. Receptor-mediated endocytosis is the most common mode of vector and toxin internalization.^(31,32) Rational cell-specific vector design for gene therapy and immunotherapy thus requires development of MAbs directed to cell surface antigens, such as p185^{HER-2}, expressed by target cells.^{16,18,33} Furthermore, the internalization potential of these MAbs should be assessed prior to retargeting of gene therapy or immunotherapy vectors to ensure efficient delivery of genes or toxins to subcellular sites of action. Here we used a radioimmunoassay to identify two novel MAbs, which were internalized after binding a cell surface receptor and, therefore, may be useful for delivery of genes and/or toxins into target cells via an endolysosomal pathway.

Some MAbs mimic the effects of ligands in experimental systems. MAbs directed against the rat p185 receptor trigger receptor dimerization, phosphorylation, and downregulation of rat p185, whereas monovalent Fab' have not.⁽³⁴⁾ Another p185^{HER-2} MAb was shown to undergo receptor-mediated endocytosis by electron microscopy following capping at membranes of p185^{HER-2} overexpressing NIH 3T3 cells.⁽³⁰⁾ Intracellular trafficking was previously documented by immunogold detection of other MAbs in membrane-bound organelles.²⁹ Although this assay has been used to determine the subcellular fate of the murine precursor to the therapeutic MAb HerceptinTM, only a few other MAbs in experimental protocols for immunotoxin therapies or targeted gene therapies have been tested for internalization.^(30,35,36) Furthermore, the radioimmunoassays used did not identify evidence of endolysosomal trafficking. Here, a rapid radioimmunoassay has been tested for its utility in determining the postbinding subcellular fates of novel MAbs to the p185^{HER-2} receptor.

Evidence of 8H11 and 10H8 antibody trafficking through receptor-mediated endocytosis and degradation by lysosomal

sorting was demonstrated. TCA-soluble fractions of supernatant counts were found in 8H11 and 10H8 assays. No TCA-soluble counts were found in the supernatants of NIH 3T3 pulsed control cells. The radioimmunoassay therefore delineated the fates of antibodies to be assessed for receptor-mediated endocytosis.

Future retargeting of vectors and toxins can be improved by characterizing the postbinding fates of the engineered constructs. In particular, nonviral antibody vectors that lack viral endosome escape domains have been fused with viral or toxin polypeptides known to disrupt endosomal membrane integrity.^(15,32,37-39) Endosomal routing is critical for these added domains to mediate cytoplasmic entry of molecular conjugates. Because divalency is required for antibodies to trigger receptor dimerization and internalization, divalent binding sites may be needed in at least some engineered vectors to ensure receptor-mediated endocytosis.^(34,40) Therefore, the radioimmunoassays described may be useful in predetermining antibodies and antibody-based vectors for dimerization and subsequent receptor-mediated endocytosis.

To develop effective MAb-based therapies, determining the internalization potential and, particularly, the trafficking of MAb-antigen to an endolysosomal pathway is important. After altering targeting specificities, prokaryotic and plant toxin-MAb conjugates and targeted viral and nonviral conjugates should be assessed for retention of internalization potential. Hence, the radioimmunoassay which determined the internalization of MAbs 8H11 and 10H8 could be used to assess the subcellular fates of newly developed MAbs against cancer-specific surface antigens to improve internalization-dependent therapies.

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